

Applications of Capillary Supercritical Fluid Chromatography-Supercritical Fluid Extraction to Natural Products

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Abstract

Capillary supercritical fluid chromatography (SFC) is uniquely suited for the separation of complex mixtures of natural products having nonpolar to moderately polar components with a molecular weight range of 100–1000 Daltons. This study describes several generic separation problems where SFC, or SFC coupled with micro-supercritical fluid extraction (SFE), can be utilized to the analyst's advantage. For example, capillary SFC can be used to separate the analytes of interest from a relatively nonvolatile sample matrix without resorting to sample preparation prior to chromatography. Other applications of capillary SFC include the separation of reaction products from higher molecular weight starting materials and the deformation of commercial products. Capillary SFC, when coupled with micro-scale SFE also permits the characterization of small samples, such as portions of single seeds and extractables from single, live insects.

Introduction

The development of supercritical fluid chromatography over the past ten years has given rise to a plethora of application studies designed to demonstrate the uniqueness of this versatile analytical tool. The advantages of SFC over gas or high-pressure liquid chromatography have been noted (1,2) for specific types of samples, such as oligomeric polymer mixtures (3) or complex mixtures of oleophilic components that can be readily solubilized in supercritical CO₂ (4). SFC permits the separation and analysis of thermally labile, high molecular weight solutes (5) without resorting to sample derivatization. In addition, SFC retains many of the superior separation efficiency characteristics of gas chromatography while exploiting the variable density-based solvent power of the mobile phase to mimic high-performance liquid chromatographic selectivity.

Over the past two years, we have applied SFC for the characterization of a number of complex natural samples of agricultural origin and as a survey tool to assess the feasibility of performing larger scale supercritical fluid extractions (SFE) on these matrices. In the process, we have identified several unique applications of the technique, which may be classified as general solutions to the separation of complex mixtures containing

natural products. Such a classification differs from a division of SFC separations based upon sample types (6).

In this paper we intend to show that capillary SFC is an excellent technique for separating these complex mixtures and that, when coupled with supercritical fluid extraction, it permits the characterization of micro samples. The separations by SFC reported in this study were initiated to solve problems in agricultural chemistry but should have wide application in other technological fields. The methods demonstrated in these studies are applicable to nonpolar and moderately polar solutes with molecular weights between 100 and 1000 Daltons. These criteria assure sample dissolution in the mobile phase, supercritical CO₂, which was maintained at varying pressures up to 400 atm.

Experimental

A model 501 supercritical fluid chromatograph (Lee Scientific) controlled by an IBM PC-AT computer was used throughout the course of this investigation. Chromatograms were recorded on an Omniscrite recorder (Houston Instruments) while hard copy of the CRT control screen was dumped to a Panasonic KX P1091 printer. High purity grade carbon dioxide for SFC was obtained from Scott Specialty Gases and was further purified by passage through a trap containing alumina, basic-Brockmann I (Aldrich Chemical), activated at 450°C overnight. Unless otherwise stated, all injections were performed with a Valco injection valve, cooled to approximately –10°C, with an internal injection cavity of 200 nL. An injection sequence of 12 s permitted the entire contents of the loop to be transferred to the capillary column.

Detection was accomplished with a flame ionization detector operating at 350 or 375°C. Back pressure on the capillary column was provided by using a frit restrictor (Lee Scientific) tailored for operation with 50- μ m i.d. capillary columns. For most of the reported separations, initial linear velocities through the column were between 2.0 and 4.0 cm/sec. Samples were dissolved in *n*-hexane (1–10 wt %), with the occasional addition of 1–2 drops of methylene chloride or acetone to remove any trace of sample turbidity.

The separations reported in this study were accomplished on one of three capillary columns: SB-Methyl (10 m \times 50 μ m i.d.), SB-Octyl (15 m \times 50 μ m i.d.), and SB-Biphenyl-30 (10 m

$\times 50 \mu\text{m}$ i.d.), all having stationary phase film thicknesses of $0.25 \mu\text{m}$ (Lee Scientific). The SB-Methyl column stationary phase consisted of a cross-linked methylpolysiloxane polymer while the SB-Octyl column utilized an *n*-octylmethylpolysiloxane phase. Similarly, the SB-Phenyl employed a 50:50 mixed phenylmethylpolysiloxane bonded phase. The operating column temperatures are noted on the relevant chromatograms, but in general were either 100 or 120°C . Several time-based density programs were used to effect the reported separations, each consisting of an initial density hold for 10–15 min followed by a density programming rate of $0.005\text{--}0.01 \text{ g/mL/min}$ with a final density hold for 10–15 min. Typical starting densities were $0.15\text{--}0.28 \text{ g/mL}$, with the program terminating at mobile phase densities of $0.66\text{--}0.76 \text{ g/mL}$. Analysis times were approximately 90 min under these instrument programming conditions. Density programs of shorter duration were also utilized after surveying the complexity of the natural product mixture by the programs described above.

On-line supercritical fluid extractions were performed with the aid of a Lee Scientific supercritical fluid extraction injection accessory mounted on top of the chromatograph oven (7). Small microcells ranging in volume from 0.3 to 3.5 cc were used to contain the samples for extraction. Extraction density or pressure, extraction time, and cell temperature were controlled by the pump software program in the external computer. Specific values of these experimental parameters as applied to the extraction of specific samples are noted on the relevant chromatograms. After initiation of the extraction program, the supercritical fluid flowed for a specified time through the extraction cell, then through a restrictor orifice into a cryofocusing tee cooled by carbon dioxide from an external source. Upon initiation of the chromatographic program, the extracted solutes were defocused by removal of the coolant and swept as a sharp band onto the capillary column.

Results and Discussion

Four generic analysis problems were examined by SFC and SFE-SFC while using the above described experimental parameters. The first class of separations involved the use of SFC for isolating a target analyte from a relative nonvolatile sample matrix. In this case, SFC allows the analyst to utilize density or pressure programming to separate the analytes of interest from the background matrix components, which are swept out of the column at the end of the program. This approach saves sample workup prior to analysis, since separation of the analyte from interfering background components is done during the analysis step.

Another general application of SFC is for the examination of reactant-product mixtures in which the products are derived from a higher molecular weight substrate. The capability of SFC to separate an unusually wide molecular weight range of compounds allows simultaneous monitoring of both complex reactant and product in mixtures without resorting to multiple analytical techniques. This approach provides the synthetic chemist with an overall graphic picture of his reaction components as well as the degree of conversion. Conversely, SFC, within certain molecular weight limits, permits analysis of reactant mixtures yielding higher molecular weight products in one chromatographic run. This latter approach has been amply illustrated in the literature for polymerization reactions (8, 9).

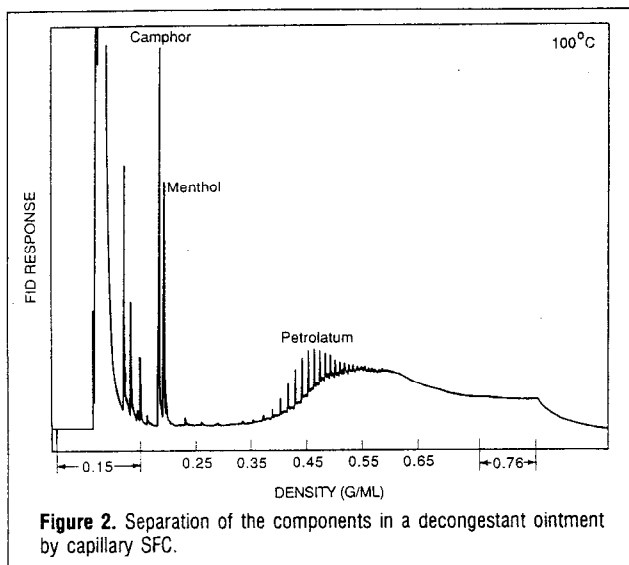
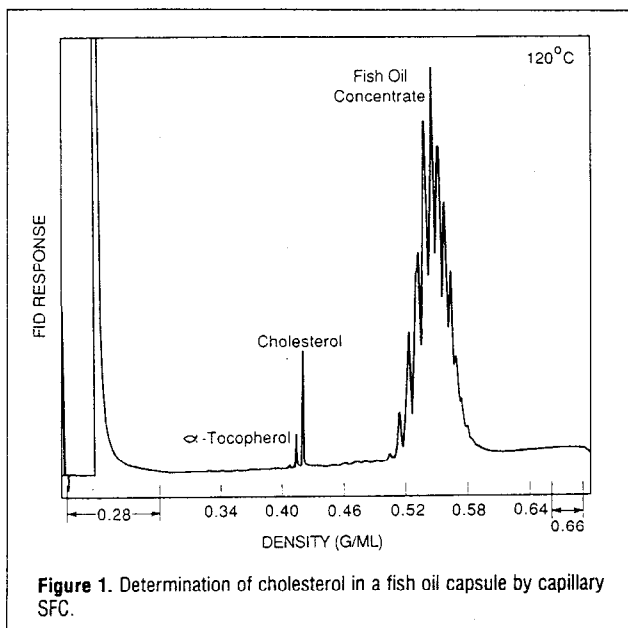
A third area of potential use for SFC is in the deformation of commercial products, particularly those formulations that contain oleophilic compounds of natural origin. In this case, the resultant SFC chromatographic profile provides a visual display of the components in the product to the formulation chemist. There are limitations to this approach when using pure carbon dioxide as the carrier gas, because very polar components may not partition into the mobile phase upon injection, or they may be only sparingly soluble in the injection solvent. As a general rule, compounds that can be solubilized from their respective product formulations in *n*-hexane or methylene chloride containing small amounts of polar organic cosolvents will readily partition into the supercritical fluid mobile phase upon injection of the sample into the chromatograph.

Finally, capillary SFC can be combined with small extraction cells to provide some unique information on particular matrices. The use of SFE in this coupled approach adds an extra dimension of fractionating power to the overall assay of complex natural product mixtures. This technique is particularly prone to contamination and requires caution on the analyst's part in achieving a chromatographic profile that is representative of the sample matrix. In addition, sample sizes for these minaturized extractions tend to be small (under one milligram in some cases), in order to assure that a complete extraction can be achieved in a reasonable time period. This is particularly the case for sample matrices with a high level of extractable material, such as lipid-rich substrates.

Target analyte isolation from a nonvolatile sample matrix

An example of analyte isolation from a relatively nonvolatile background is provided in Figure 1 where the cholesterol content of a fish oil capsule has been determined by SFC. In this assay, the contents of a single capsule (approximately 1000 mg) were emptied into the injection solvent, *n*-hexane, by inserting a needle into the gelatin capsule to permit extrusion of the oil into the solvent. The oil-laden solution was then directly injected into the SFC and separated with the density program inscribed on the time axis. Two peaks were found to elute within the programmed density interval corresponding to $0.40\text{--}0.42 \text{ g/mL}$. The identities of the two peaks were found by spiking the above solution with known amounts of α -tocopherol and cholesterol and separating the two spiked solutions with the same program. As shown in Figure 1, the natural antioxidant, α -tocopherol (Vitamin E), elutes slightly before the spiked cholesterol profile. The level of the cholesterol in the unspiked solution was found to be 5 mg and yielded an elution peak height just slightly larger than that recorded for α -tocopherol. The background matrix of fish oil triglycerides was not resolved under these programming conditions on the SB-Methyl column at 120°C . They could, however, be eluted off the capillary column, thereby avoiding any tedious sample preparation step, such as gel permeation chromatography, prior to final chromatographic analysis. Better resolution of fish oil triglycerides by SFC has been reported in the literature (10), but this was not the objective in the current study.

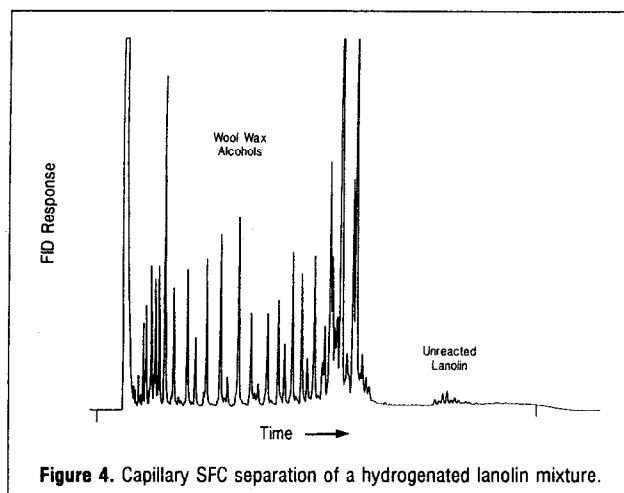
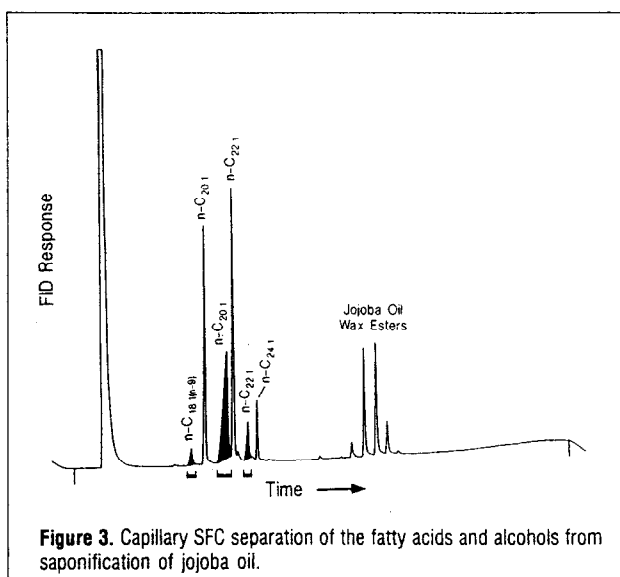
A similar approach was also employed to determine the medicinal ingredients in a decongestant ointment containing a petrolatum base. As shown in Figure 2, an extended density program of the chromatographic mobile phase at 100°C on the SB-Methyl capillary column resolved the two active components, camphor and menthol, from the higher molecular weight base. Such a separation illustrates the capability of SFC to effect a separation between very volatile components normally analyzed



by gas chromatography and nonvolatile, high molecular weight, wax-like components. The oligomeric components comprising the background matrix were separated by the described density program, eluting within the density range of 0.25–0.65 g/mL. The high resolution recorded for the matrix peaks may be sacrificed in lieu of a more rapid chromatographic assay by employing a higher density program rate after the hold period.

Reactant-product mixture separation

As noted previously, capillary SFC is an excellent technique for examining reactant-product mixtures derived from natural substrates. In our research, we have utilized SFC to assess the extent of conversion of agriculturally-derived materials such as lanolin and jojoba oil. Figure 3 is a chromatogram of jojoba oil (average molecular weight = 606) which was saponified to produce the component long chain fatty acids and alcohols. The chromatographic profile was obtained on an SB-Methyl column with the program reported for Figure 1. The wax esters of jojoba oil yielded a mixture of unsaturated fatty acids and alcohols ranging in chain length from C_{18} to C_{24} . The shaded product



peaks are the acid moieties, which give fronting peak profiles on the nonpolar bonded and cross-linked polymethylsiloxane stationary phase. The product peaks are well separated from the starting substrate, jojoba oil, which is characterized by its wax ester distribution representing major chain lengths from C_{18} to C_{24} . The presence of unreacted jojoba oil in the reaction mixture was verified by injecting an *n*-hexane solution of the starting oil. Matching retention times were recorded for each of the jojoba oil components, testimony to the excellent reproducibility of the density program from the computer-controlled pump. It should be noted that separation of this reaction mixture by capillary SFC was achieved at a relatively low temperature, 120°C, and did not require the formation of derivatives or ultra high analysis temperatures as reported by other researchers (11).

A similar approach can be used to characterize reaction mixtures of lanolin, a complex mixture of high molecular weight esters and polyesters (average molecular weight = 790–880) derived from the sebaceous glands of sheep. Exhaustive hydrogenation of lanolin produces only alcohols as reaction products (12) and these compounds can be nicely separated on an SB-Octyl column, as shown in Figure 4, with the density program utilized for Figure 1. The capillary SFC profile in Figure

4 indicates that there is little residual lanolin left upon completion of the reaction.

Deformulation of commercial products

Capillary SFC is an excellent technique for deformulating commercial products that contain nonpolar to moderately polar components that can be dissolved in the chromatographic mobile phase. By applying the proper density or pressure program, the solubilized product components can be separated on the resultant chromatogram, thereby providing the product formulator with a visual display of the key ingredients in the commercial formulation. In the case of pharmaceutical or cosmetic products, there are components derived from natural sources that yield complex chromatographic elution patterns. These moieties can best be resolved and identified by capillary chromatographic techniques.

An example of this method is shown in Figure 5 where the components in an analgesic ointment have been determined by capillary SFC. This pharmaceutical preparation was dissolved in *n*-hexane with gentle warming and directly injected onto an SB-Methyl column and then subjected to density programming with CO₂ as the mobile phase. The two major medicinal com-

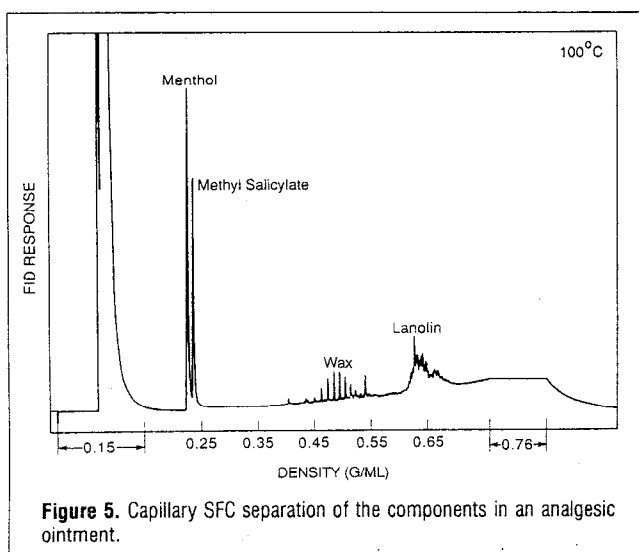


Figure 5. Capillary SFC separation of the components in an analgesic ointment.

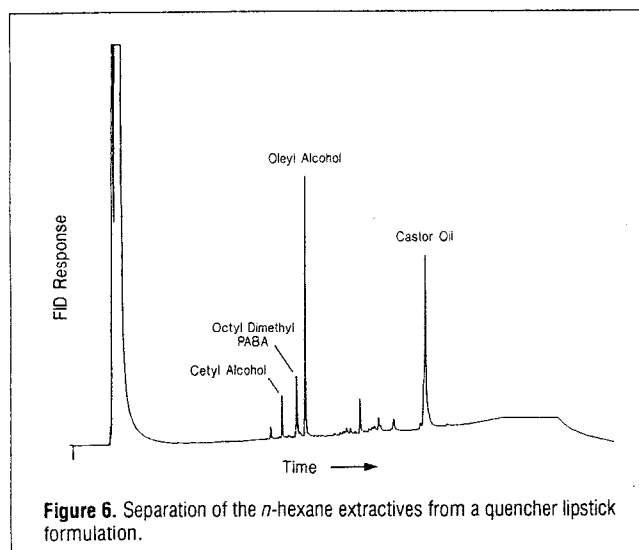


Figure 6. Separation of the *n*-hexane extractives from a quencher lipstick formulation.

ponents of the ointment were found to elute slightly before achieving a program density of 0.25 g/mL. These compounds were followed by a symmetrical distribution pattern of a synthetic wax, which eluted at a program density between 0.45 and 0.55 g/mL. The identification of this component was based on its elution position on the density axis of the chromatogram and the distribution pattern of the wax constituents. In addition, comparisons were made with both natural and synthetic wax standards that were run under identical SFC conditions. Similarly, the unresolved profile of lanolin, a common ingredient in this type of preparation, was identified at a program density of approximately 0.65 g/mL.

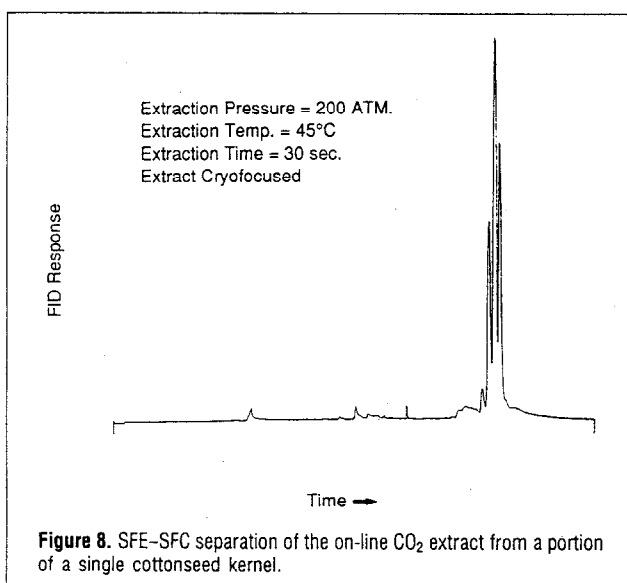
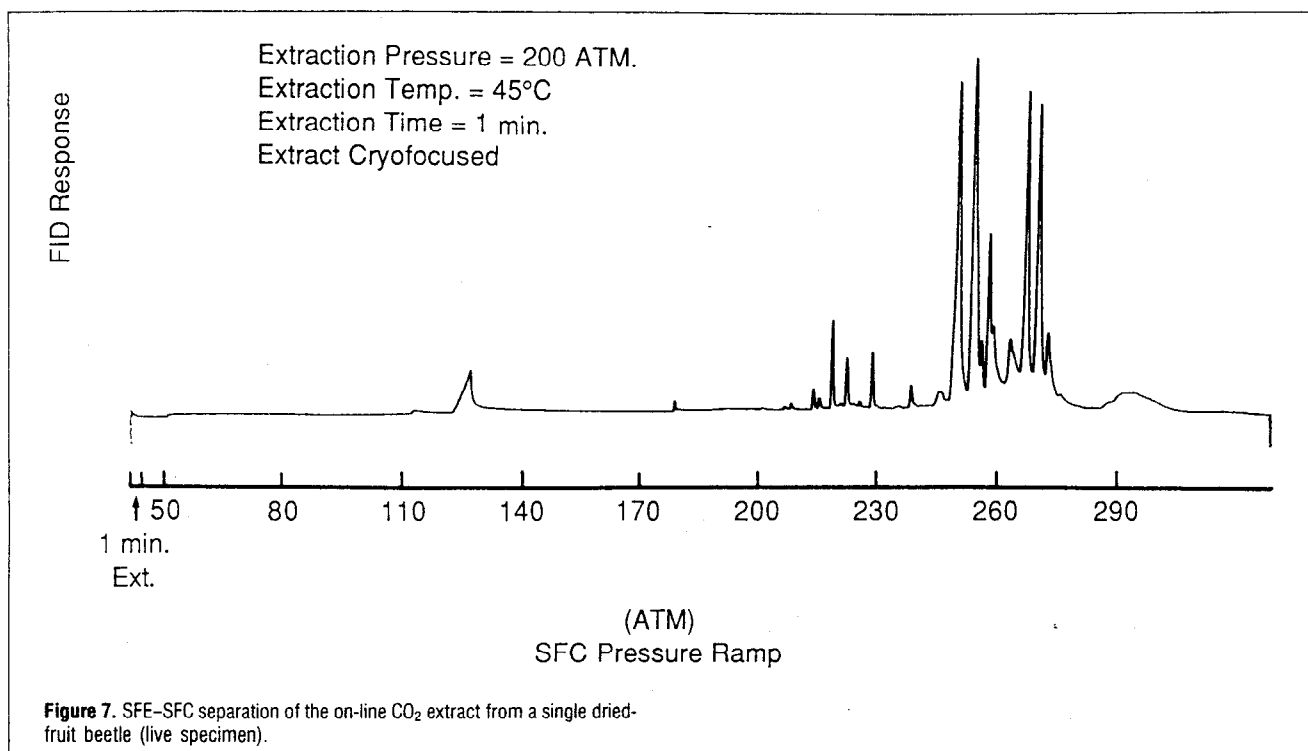
Cosmetic formulations can be analyzed in a similar manner. Many products such as lipsticks and lip balms are quite rich in oleophilic ingredients that can be solubilized in *n*-hexane and the supercritical carbon dioxide mobile phase. Addition of *n*-hexane to pigmented products results in precipitation of the micronized lakes or pigments, allowing direct injection of the supernatant layer into the SFC. The resultant SFC profile of the *n*-hexane extract of a "quencher" lipstick is shown in Figure 6. This chromatographic separation was obtained with the same mobile phase density program given for Figure 5. Prominent peaks in the chromatogram were identified by running standards of the compounds that were known to be in the lipstick formulation. These peaks were confirmed to be castor oil, oleyl and cetyl alcohols, and octyl dimethyl PABA (a common antioxidant used in cosmetic formulation), respectively. Capillary SFC of other lipstick formulations has yielded distinct chromatographic profiles that are specific to a particular commercial product, allowing the establishment of a library of capillary SFC data on specific cosmetic products.

Coupled SFE-SFC on natural substrates

Micro-scale SFE in combination with capillary SFC provides yet another tool for the analyst to examine natural substrates. As noted earlier, this technique is particularly sensitive to the presence of lipophilic extractables, and the extraction can also be affected by the moisture level of the sample matrix (13). Although considerable progress has been made in optimizing analytical scale SFE (14), it is still necessary for the analyst to determine the proper extraction time, pressure, and sample size for a particular sample matrix. Despite these current limitations, we have found micro-scale SFE to be of value in characterizing very small samples related to certain agricultural problems.

An example of such a problem is the analysis of cuticular hydrocarbons and waxes on the surface of insects. The size of the microextractors mentioned previously is adequate for the SFE of microtome-cut insect body parts or the entire insect in some cases. Figure 7 is the profile obtained from the supercritical fluid extraction of a live driedfruit beetle (*Carcophilus hemipterus*), a pest that attacks a large number of agricultural commodities (15). The insect was placed into the extraction cell after brief refrigeration to lower its metabolic activity. The cell was then capped and inserted into the controlled-temperature extraction cell block on top of the chromatograph.

The on-line extraction, as noted in Figure 7, was performed at a CO₂ pressure of 200 atm for one minute. After cryofocusing, the extract was fractionated with the pressure program inscribed on the horizontal axis in Figure 7. The major components of the extract eluting in the latter half of the pressure-programmed run were at an elution position identical to those recorded for high molecular weight hydrocarbon wax components (16). Unfortunately, our experimental apparatus was



not configured as an SFE-SFC-MS system. Such a tandem arrangement would be of considerable value in identifying the components in the complex mixtures that often result from SFE. The skewed peak appearing earlier in the SFC density program is probably an acidic moiety, since a nonpolar SB-Octyl column was used for this separation. It should be noted that the supercritical fluid extraction was performed at the relatively low temperature of 45°C. The use of this temperature for extraction suggests that SFE may have applicability for the isolation and identification of pheromones whose molecular structures could be altered by high temperatures or oxidative analysis conditions.

Another interesting application of micro-SFE is the characterization of single seeds with respect to their triglyceride and

fatty acid compositions. Such information is of value to scientists involved in seed hybridization and genetic engineering of plants. Ideally, sample workup and analysis should require minimal quantities of single seeds, because such moieties can then be planted after small portions are removed for analysis. Figure 8 is the supercritical fluid chromatographic profile taken after SFE on less than one milligram of cottonseed kernel. An extraction pressure of 200 atm at 45°C for 30 s was used to obtain the profile in Figure 8. A density-based analysis program identical to that used in Figure 7 (the column analysis temperature was 100°C) was used to perform the separation. Unfortunately, column overload occurred under these extraction conditions as evidenced by the incomplete resolution of the principal triglycerides (C₅₀-C₅₄) of cottonseed oil. The difficulty in adjusting extraction conditions for substrates with a high lipid content has been noted by Hawthorne (17), but this problem can be corrected by employing a smaller sample size in the microextractor or by using a larger diameter capillary column.

Conclusions

The results presented here show that capillary supercritical fluid chromatography is an appropriate technique for characterizing natural product mixtures comprised of nonpolar to moderately polar components encompassing a wide molecular weight range. In many cases, the use of capillary SFC can eliminate tedious sample preparation procedures prior to chromatographic analysis as well as permit direct injection of product formulations containing components that can be readily solubilized in the chromatographic mobile phase. Micro-scale supercritical fluid extraction coupled to capillary SFC permits the simultaneous extraction and analysis of small biological samples that would be difficult to characterize by conventional analytical methodology.

Acknowledgment

Dr. Robert J. Bartelt kindly provided the driedfruit beetle specimens.

Disclaimer

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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Manuscript received November 9, 1989;
revision received November 18, 1989.